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Infection with *Bartonella henselae* in a Danish Family

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***Bartonella* species constitute emerging, vector-borne, intravascular pathogens that produce long-lasting bacteremia in reservoir-adapted (natural host or passive carrier of a microorganism) and opportunistic hosts. With the advent of more sensitive and specific diagnostic tests, there is evolving microbiological evidence supporting concurrent infection with one or more *Bartonella* spp. in more than one family member; however, the mode(s) of transmission to or among family members remains unclear. In this study, we provide molecular microbiological evidence of *Bartonella henselae* genotype San Antonio 2 (SA2) infection in four of six Danish family members, including a child who died of unknown causes at 14 months of age.**

Historically, infection of immunocompetent individuals with *B. henselae*, the sole or primary cause of cat scratch disease (CSD), was medically categorized as typical or atypical CSD cases (1, 2). Recently, the use of the more inclusive term "bartonellosis" has been recommended so as to encompass bacteremic infections with the 14 *Bartonella* species that now have been implicated as human pathogens (3, 4). Despite a large body of scientific literature relative to CSD, an individual cat has rarely been implicated in the transmission of *B. henselae* to more than one person within the same household (5, 6); however, people in a household where one person has CSD are more likely than others to have been exposed serologically or to also have had CSD (7). Therefore, cat flea (*Ctenocephalides felis*) and cat scratch transmission may cause bartonellosis among multiple family members. Currently, cat fleas, which can infest cats, dogs, coyotes, raccoons, and numerous other small mammals, remain an underappreciated vector for human *Bartonella* species (*Bartonella clarridgeae*, *Bartonella henselae*, *Bartonella koehlerae*, and potentially *Bartonella quintana* and *Bartonella bovis*) infections (1–3, 6).

With the advent of more extensive serological and more sensitive diagnostic modalities, including PCR and combined enrichment blood culture-PCR, case reports describing *Bartonella* sp. infections involving more than one family member have been published; however, the route(s) of transmission remains unclear or could not be established on the basis of the medical histories. In the United States, infections with *B. henselae* and/or *Bartonella vinsonii* subsp. *berkhoffii* genotype II were documented in two of five (8), four of four (9), and three of three (10) family members, respectively. In Australia, the male partner of a woman who was infected with *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* subsequently was found to be infected with *B. koehlerae* (11). Infection with *B. henselae* also was documented in two of four family members from the Netherlands (12). The symptoms associated with bartonellosis in nonimmunocompromised patients often are subtle and nonspecific and have only recently been characterized in part. Therefore, most diagnosticians and clinicians would not intuitively pursue the possibility of infection with one or more *Bartonella* spp. in multiple family members. Here, we document infection with the same *B. henselae* genotype SA2 in four of six family members from Denmark, including a son who died of undetermined causation at 14 months of age.

MATERIALS AND METHODS

Study participants and samples tested. The years of birth for the father, mother, daughter I, daughter II, son, and daughter III were 1961, 1964, 1990, 1992, 1999, and 2003, respectively. Based upon her review of publications from our research group, the mother of this Danish family contacted the corresponding author to request testing for evidence of *Bartonella* infection in her deceased 14-month-old son who was diagnosed with sudden infant death syndrome (SIDS), in a 7-week-old fetus aborted in 2001, and in the five surviving family members, which included the father, mother, and three daughters. Individual tissues from the 14-month-old deceased son were extracted and tested on different days between 20 and 25 September 2012. After infection with *B. henselae* was verified in the son, the surviving family members (father, mother, daughters I, II, and III) were tested between November 2012 and June 2013. Tissues from the aborted fetus were tested in March 2014, and stored blood spots from the four children were tested in July 2014. EDTA-anti-coagulated blood, serum samples, tissues, and blood spots from the family were shipped from Denmark to the Intracellular Pathogens Research Laboratory (IPRL), College of Veterinary Medicine, North Carolina State University, where all *Bartonella* testing was performed. Each surviving family member completed a standardized questionnaire that had been used in our previous research studies (13, 14). With the permission of the parents, 3.2-mm punches from dried blood spot samples (equaling 3 μl of whole blood) obtained from the heel 48 to 72 hours after the birth of all four children were provided by the Danish Center for Neonatal Screening (see Acknowledgments) for *Bartonella* PCR. Thirty-micrometer formalin-fixed, paraffin-embedded tissue sections (heart, thymus, brain, liver, and spleen) from the 14-month-old deceased son and placental tissues from a 7-week-old aborted fetus were tested for *Bartonella* spp. by PCR. Collection and analyses of these data were conducted in conjunction with North Carolina State University Institutional Review Board approval (IRB no. 1960).

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Serological analyses. As previously described (11–14), *Bartonella vinsonii* subsp. *berkhoffii* genotypes I, II, and III, *B. henselae* Houston-1 strain (H-1), *B. henselae* (SA2 strain), and *B. koehlerae* antibodies were determined by indirect immunofluorescence antibody assay (IFA) using fluorescein-conjugated goat anti-human IgG (Pierce Biotechnology, Rockford, IL). Briefly, *Bartonella* organisms of feline (*B. koehlerae* [NCSU 09FO-01] and *B. henselae* H-1 [NCSU 93FO-23], *B. henselae* SA2 [NCSU 95FO-099]) and canine (*B. vinsonii berkhoffii* genotype I [NCSU 93CO-01], II [NCSU 95CO-08] and III [NCSU 06CO-01]) origins were passed from 5% sheep blood agar into cell lines to obtain antigens for IFA testing as described previously (15). Heavily infected cell cultures were spotted onto 30-well Teflon-coated slides (Cel-Line/Thermo Scientific, Waltham, MA), air dried, acetone fixed, and stored frozen until tested. Serum samples were diluted in phosphate-buffered saline (PBS; pH 7.4) containing normal goat serum, 0.05% Tween 20, and 0.5% powdered nonfat dry milk to block nonspecific antigen binding sites. Sera were tested at dilutions of 1:16 to 1:8192. A cutoff IFA titer of ≥1:64 was used to define a seroreactive titer.

DNA extraction, PCR assay, and DNA sequencing. A previously described approach that combines PCR amplification of *Bartonella* species DNA from blood, serum, and *Bartonella* alphaproteobacterial growth medium (BAPGM) blood cultures at 7 and 14 days of incubation was used (13, 14, 16, 17). DNA was extracted from 200 μl of EDTA blood and serum and from 200 μl of 7- and 14-day BAPGM enrichment blood cultures, using an automated BioRobot symphony workstation and MagAttract DNA blood kit (Qiagen, Valencia, CA). To check for potential contamination during processing, we simultaneously processed a noninoculated BAPGM culture flask in the biosafety hood in an identical manner for each batch of patient blood and serum samples tested. For PCR, negative controls were prepared by using 5 μl of DNA from the blood of a healthy dog.

Genomic DNA from the deceased son's 30-μm formalin-fixed, paraffin-embedded tissue sections (heart, thymus, brain, liver, and spleen), cumulative tissues from the 7-week-old aborted fetus and from the dried blood spot samples from all four children was extracted using a Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions. To avoid contamination, DNA was extracted from each tissue type independently at different time points. Elution buffer was used as a negative control and processed simultaneously to ensure that extraction buffers and reagents were not contaminated with *Bartonella* DNA.

Bartonella DNA was amplified using conventional *Bartonella* genus-specific and *B. koehlerae* species-specific PCR primers targeting the 16S-23S intergenic spacer region (ITS) as previously described (13, 17). *Bartonella* genus PCR was performed using either oligonucleotide 425s (5′C CGGGGAAGGTTTTCCGGTTTATCC3′) or 325s (5′ CCTCAGATGAT GATCCCAAGCCTTTTGGCG 3′) as forward and reverse primers, in combination with oligonucleotide 1000as (5′ CTGAGCTACGGCCCCT AAATCAGG 3′), rendering an expected amplicon of 600 to 650 bp (bp) or 700 to 750 bp, respectively. Similarly, *B. koehlerae* species-specific PCR was performed using oligonucleotides Bkoehl-1s (5′ CTTCTAAAATAT CGCTTCTAAAAATTGGCATGC 3′) and Bkoehl1125as (5′ GCCTTTTT TGGTGACAAGCACTTTTCTTAAG 3′) as forward and reverse primers, respectively, with an expected amplicon size of 600 bp. *Brucella* species PCR was performed using genus-specific primers, which includes *Brucella*ITS200s (5′ GGATTTATCCGGATGATCCTTCTCCAT 3′) and *Brucella*ITS810as (5′ CAAGCTTCTTGCGACATCAACTCTTCAG 3′) as forward and reverse primers, respectively. These primers amplify 562 bp of the 16-23S ITS region of the *Brucella* genus.

Amplification was performed in a 25-μl final volume reaction mixture containing 12.5 μl of MyTaq premix (Bioline USA Inc., Taunton, MA), 0.2 μl of 100 μmol/liter of each forward and reverse primer (IDT DNA Technology, Coralville, IA), 7.3 μl of molecular-grade water, and 5 μl of DNA from each sample tested. Amplification was performed using an Eppendorf Mastercycler EP gradient (Eppendorf, Hauppauge, NY) using 0.01 pg/μl of *B. henselae* SA2 genomic DNA as the PCR positive control for *Bartonella* genus-specific 16-23S ITS elements, whereas 0.01 pg/μl of *B. koehlerae* DNA was used as the positive control in the *B. koehlerae* species-specific PCR. Similarly, a *Brucella suis* plasmid at a concentration of 10 copies/μl was used as the positive control for the *Brucella* PCRs. Negative controls included liquid media (BAPGM), sodium phosphate glutamate buffer (pH 7.0), and molecular-grade water. The thermocycling conditions consisted of initial denaturation of 95°C for 2 s, followed by 55 cycles with denaturation at 94°C for 15 s, annealing at 68°C for 15 s, and extension at 72°C for 18 s. PCR products were analyzed by 2% agarose gel electrophoresis. Positive amplicons were sequenced (Genewiz, Inc., Research Triangle Park, North Carolina) to identify the *Bartonella* sp. and ITS genotype. Bacterial species and genotype were defined by examining similarities to other sequences deposited in the GenBank database using the basic local alignment search tool (BLAST; version 2.0). DNA extraction and PCR controls remained negative throughout the course of the study.

RESULTS

No comorbidities were reported in the 14-month-old deceased son, except for a high fever (103.8°F) that occurred shortly before death. All surviving family members had experienced periodic, nonspecific symptoms, such as joint pain, fatigue, fibromyalgia, and a low-grade fever that spanned various time intervals. Historically, the family members had infrequent contact with pet cats in the neighborhood and had only owned dogs with historical flea infestation prior to the birth of their first child. The family did not participate in farming or hunting activities. *B. henselae* genotype SA2 DNA was amplified and sequenced from the deceased son's formalin-fixed, paraffin-embedded heart and thymus tissues, whereas PCR from brain, liver, and spleen did not result in the amplification of *Bartonella* sp. DNA. *Bartonella* sp. DNA (data not shown) was not amplified from paraffin-embedded tissues from the 7-week-old aborted fetus or from blood spots from all four children. As no gross or histopathological abnormalities were reported at the autopsy of the deceased son (Aarhus University Hospital, Aarhus, Denmark), the pathologist rendered a diagnosis of SIDS. Based upon the initially obtained *B. henselae* SA2 PCR test results from the deceased son's paraffin-embedded heart and thymus tissues, the surviving family members were tested sequentially for serological and molecular evidence of *Bartonella* infections. In 2010, daughter I was diagnosed with Bell's palsy, and subsequently she tested negative for Lyme disease. In 2011, mother reportedly was seropositive for Lyme disease and treated with 8 weeks of azithromycin, followed by an azithromycin-minocycline combination for 8 weeks and hydroxychloroquine for 4 weeks. In 2012, mother also was tested serologically for *Bartonella* in Denmark. Her *Bartonella* IgG titer was inconclusive (1:128), and that for IgM was negative. The reference ranges for *Bartonella* serology in Denmark were IgG titers of <64 (negative), 64 to 256 (inconclusive), and >256 (positive). Subsequently, the mother was treated in Denmark for bartonellosis with rifampin (300 mg) and doxycycline (150 mg) every 12 h for 7 months, with symptomatic improvement. The remaining family members (father and daughters II and III) were never tested for Lyme disease or *Bartonella* sp. infection until they entered into this study.

The serological and BAPGM enrichment blood culture-PCR results for the father, mother, and three daughters are summarized in Table 1. *B. henselae* SA2 genotype DNA was amplified and sequenced from a 14-day BAPGM enrichment blood culture from the mother, a 7-day BAPGM enrichment blood culture from the

TABLE 1 *Bartonella* species serology and BAPGM enrichment blood culture PCR results for members of a Danish family

Family member and date (mo/day/yr)	*Bartonella* IgG serology[a]					BAPGM diagnostic platform result[b]			
			B. vinsonii subsp. *berkhoffii* genotype:			Preenrichment *Bartonella* 16-23S ITS element PCR		Postenrichment *Bartonella* 16-23S ITS elements PCR	
	B. henselae	*B. koehlerae*	I	II	III	Blood	Serum	7 days	14 days
Mother									
11/12/2012	Neg	Neg	Neg	128	64	Neg	Neg	Neg	SA2
01/14/2013	Neg	64	Neg	64	Neg	Neg	Neg	Neg	Neg
Father									
11/12/12	Neg	Neg	Neg	64	Neg	Neg	Neg	SA2	Neg
01/14/2013	64	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Daughter I									
11/12/2012	Neg	Neg	Neg	Neg	64	Neg	SA2	Neg	Neg
01/14/2013	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Daughter II									
11/12/2012	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
01/14/2013	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
06/17/2013	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Daughter III									
11/12/2012	Neg	Neg	Neg	128	Neg	Neg	Neg	Neg	Neg
01/14/2013	256	Neg	Neg	1,024	512	Neg	Neg	Neg	Neg
06/17/2013	64	Neg	Neg	256	Neg	Neg	Neg	Neg	Neg

[a] Neg, negative.
[b] DNA was extracted for PCR amplification from blood and serum and from 7- and 14-day BAPGM enrichment cultures. SA2, *B. henselae* SA2.

father, and from serum of daughter I. Despite repeated testing over an 18-month time period, *Bartonella* sp. DNA was not amplified from daughters II and III. DNA of other *Bartonella* species was not amplified from any family member, and no *Bartonella* isolates were obtained from 7- or 14-day BAPGM enrichment blood subcultures. With the exception of daughter II, all other surviving family members were seroreactive (≥1:64) to one or more *Bartonella* sp. IFA antigens. *Brucella* DNA was not amplified from the blood of any family member.

DISCUSSION

Based upon recent microbiological findings, physicians and microbiologists should be aware of the potential for familial infections with one or more *Bartonella* species. In this report and other cases reported to date (8–12), the route(s) of transmission to or among family members remains unclear. The mode(s) of transmission may vary among and within families depending on environmental, social, microbiological, and epidemiological factors. Thus, the source and route of *B. henselae* SA2 transmission among individuals within this family may have been the same or different. It is clear that most, if not all, *Bartonella* species can be directly or indirectly transmitted by an arthropod vector (fleas, keds, lice, sandflies, ticks, and biting flies) (18) among various reservoir-adapted hosts. Although less thoroughly studied, it is likely that many of the same arthropods can directly (bite transmission) or indirectly (contamination of wounds with arthropods' infected feces) transmit *Bartonella* species to humans (19). Although vector competence has not been proven, transmission of *B. henselae* to people by other arthropods, including wood louse hunter spiders (10), bull ants (20), and tropical rat mites (21), has been suspected on the basis of historical medical findings in conjunction with serological and molecular test results. In the context of European familial infections, tick transmission of *B. henselae* genotype SA2 was suspected in two of four members of a family from the Netherlands (12). In the United States, *B. henselae* H-1 was PCR amplified and sequenced from a father, daughter, and 3 of 4 cats in the household (8). This father and daughter also were infected with *B. vinsonii* subsp. *berkhoffii* (genotype II), a bacterium for which domestic dogs and wild canines are considered reservoir-adapted hosts (2, 6, 22).

Despite PCR-based documentation of *B. henselae* infection in 3 of 5 surviving family members, only the father and daughter III were periodically seroreactive to *B. henselae* by IFA testing, using both *B. henselae* SA2 and *B. henselae* H-1 antigens. Although the mechanism is not understood, seronegative *Bartonella* bacteremia is being reported increasingly in humans by researchers around the world. A cross-sectional study by Maggi et.al (13) found that 69 of 92 *Bartonella*-infected humans did not have IFA antibodies to the infecting *Bartonella* species or genotype. Although unproven, chronic intravascular infection with *Bartonella* spp. may induce a degree of immunological anergy, resulting in an undetectable level of organism-specific antibodies in naturally infected human patients, or other mechanisms may contribute to seronegativity. In contrast to the *B. henselae* serology results, the father, mother, and daughters I and III, none of whom was documented to be infected with *B. vinsonii* subsp. *berkhoffii*, had IFA antibody titers against *B. vinsonii* subsp. *berkhoffii* genotypes II and III. Efforts to specifically amplify *B. vinsonii* subsp. *berkhoffii* DNA using several genus- and species-specific primer sets were not successful. Coinfections with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* ge-

notype II have been reported in patients from the United States (9, 13). Also, 3 of 6 patients infected with *B. koehlerae* (23) were concurrently seroreactive to *B. vinsonii* subsp. *berkhoffii* genotype II antigens. Dogs naturally infected with *B. koehlerae* (24) or experimentally infected with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* genotypes I and III (15, 24) develop a species-specific antibody response with no cross-reactivity, as have humans naturally infected with *B. koehlerae* (25) or with *B. vinsonii* subsp. *berkhoffii* genotype I (26). However, the degree to which IFA can be used in natural infections to deduce causative agents among *Bartonella* species is unknown. Recently, *B. vinsonii* subsp. *berkhoffii* genotype II DNA was found in dog blood specimens and fleas removed from dogs in Florida (27), suggesting that fleas also serve as a vector for *B. vinsonii* subsp. *berkhoffii* genotype II transmission to dogs and potentially to humans. All four known *B. vinsonii* subsp. *berkhoffii* genotypes infect dogs (28), and genotypes I, II, and III have been implicated in human infections (8, 9, 13, 29). Although tick transmission has been suspected on the basis of clinical and epidemiological data (2, 18), the mode(s) of transmission has not been established for any of the four *B. vinsonii* subsp. *berkhoffii* genotypes. There is clearly a need to more thoroughly investigate the role of various arthropods as vectors for the transmission of various *Bartonella* species to humans and other non-reservoir-adapted animals.

The family in this study reported infrequent contact with pet cats and had owned dogs with historical flea infestations only prior to the birth of their first child; thus, flea and domestic animal exposures may have been a source of *B. henselae* transmission for the parents, but this seems less likely for the children. In the context of CSD, flea-infested cats around the world play an important role in scratch transmission of *B. henselae*, the primary or sole bacterial cause of CSD (1–3). After obtaining a blood meal from a *B. henselae*-infected cat, bacterial numbers increase within the flea's intestinal tract, and the bacteria remain viable in flea feces for at least 9 days (19). There is also evolving evidence to support frequent arthropod and animal exposures in veterinary workers as an occupational risk for acquiring bartonellosis (13, 30). Based upon experimental cohousing experiments (31), direct transmission from *B. henselae* bacteremic cats to noninfected cats does not occur in the absence of fleas, which suggests that salivary contact due to licking or cat bites is an unlikely source of human infection, unless flea feces containing viable *B. henselae* are present in the cat's environment. This conclusion is further supported by a PCR study that amplified *B. henselae* DNA from cat nail bed clippings or saliva only if the cat was simultaneously infested with fleas (32). Clinically, in the context of *Bartonella* sp. transmission to people, it is important to determine if a patient, pet, or wild animal within the environment has a history of *C. felis* infestations. From a veterinary preventive health perspective, the use of parasiticides to prevent flea infestations among pets is of substantial public health importance.

Although vectors are thought to be the most frequent source of *Bartonella* sp. transmission, other modes of transmission have been implicated. In this study, *B. henselae* genotype SA2 DNA was amplified and sequenced from the 14-month-old son's postmortem paraffin-embedded tissue (heart and thymus) sections. In a previous study from the United States, both *B. henselae* SA2 and *B. vinsonii* subsp. *berkhoffii* genotype II were PCR amplified retrospectively and sequenced from postmortem tissues from an 8-day-old twin sister who died of a congenital heart defect. The same species and genotypes also were PCR amplified and sequenced from blood or BAPGM enrichment blood cultures from the mother and twin son that were obtained 10 years after the sister's death (9). As the blood spots obtained from all four children were PCR negative, it is unlikely that the *B. henselae* infections found in daughter I and the 14-month-old deceased son occurred *in utero*. Although *B. henselae* genotype SA2 DNA was not successfully amplified from paraffin-embedded 7-week-old aborted fetal tissue or the blood spots obtained at birth, it is possible that these findings represent false-negative PCR results caused by blood spot storage, the small quantity of blood available for DNA extraction from the blood spot, or formalin fixation and DNA cross-linking of the aborted fetal tissues. A study from Israel found that 6 of 8 babies born to mothers with a history of cat scratch disease during their pregnancies remained healthy following birth and during a median 4.5-year follow-up period (33). Therefore, if *in utero* transmission occurs, this mode of transmission presumably is infrequent; however, this is an area in which additional research is needed.

On a comparative One Health basis, as summarized in recent reviews (34–36), various *Bartonella* species cause vasoproliferative lesions in animals and people, granulomatous inflammation in dogs and people, and endocarditis in cats, cows, dogs, and people. However, the spectrum of disease manifestations that constitutes bartonellosis remains medically and microbiologically unclear. For this study, the corresponding author was contacted by the mother because, for many years, all family members had experienced nonspecific symptoms, including joint pain, fibromyalgia, and fatigue spanning different intervals of time. Whether occult *B. henselae* infection contributed to these symptoms could not be established retrospectively. In recent years, there has been growing interest in the role of *Bartonella* species as a cause of persistent bloodstream infections in nonimmunocompromised patients who frequently report chronic, often intermittent and nonspecific symptoms (13, 14, 37). There is also case-based evidence that chronic *Bartonella* sp. bloodstream infections may predispose the subject to rheumatological and neurological disease manifestations (14, 38). Clearly, in conjunction with the availability of more sensitive diagnostic testing modalities, there is a serious need for prospective, case-control studies with sequential testing over time to clarify the role of *B. henselae* and other *Bartonella* species as a cause or cofactor for chronic, nonspecific disease manifestations. In conclusion, difficulties in studying *Bartonella* species risk factors, pathology, and potential treatment options lie in the bacterium's versatile host affiliation, its ability to infect a variety of host cells, the ability to be transmitted by multiple routes and arthropod vectors, and its ability to seemingly induce a spectrum of disease in a variety of animal species that ranges from self-limiting and benign to chronic and complex. Variability in *Bartonella* strain virulence, in combination with the nuances of the host immune response (influenced by various nutritional, genetic, and other medically relevant factors), appears to create an unpredictable pattern of disease expression within or across infected animal species. Increased appreciation for the evolving understanding of the complexity of clinical presentations, in conjunction with the challenges associated with diagnosis and medical management of bartonellosis, hopefully will benefit future patients.

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